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Technical Notes

Overcoming Backpressure Problems during Solid-Phase Synthesis of Oligonucleotides

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Abstract:

During solid-phase synthesis of oligonucleotides in packed-bed reactors a substantial pressure build-up is observed during acid deprotection and subsequent solvent washing as the length of the support-bound oligonucleotide increases. Maintaining high flow rates of reagents and wash solvents is crucial to achieving the necessary short acid-exposure times during these steps. Exceeding the maximum system pressure limit, especially likely to occur in the latter part of the synthesis, may lead to shutdown of the automated synthesis. Addition of inert filling materials (e.g., glass microspheres, quartz sand) to the polymeric polystyrene-based solid support reduces pressure build-up, allowing automated synthesis to go to completion without reducing flow rates or manual intervention.

Oligonucleotides (PO-oligos) and their analogues are finding widespread utility in diagnostics, molecular biology, and as therapeutic agents. DNA and RNA analogues, especially phosphorothioate oligonucleotides (PS-oligos) in which one nonbridging oxygen of the natural internucleotide phosphate diester linkage is replaced by sulfur, are emerging as broadly useful drugs operating through antisense mechanisms of action.[1] Automated solid-phase synthesis is the first-choice synthetic method for preparation of PO- and PS-oligos in all phases of drug discovery, development and production. It involves sequential coupling of activated monomers to an elongating oligomer, the 3′-terminus of which is covalently attached to the solid support. The chemical synthesis process to assemble the oligonucleotide is carried out using a DNA synthesizer that delivers specified volumes of reagents and solvents to and from the solid-phase reactor without isolation of intermediate oligonucleotides. The flow of reagents and solvents is regulated by a series of computer-controlled valves and pumps. The solid-phase approach allows for easy purification of the support-bound reaction products at each step in the synthesis by solvent washing of the solid support. Commercially available 5′-*O*-(4,4′-dimethoxytrityl) (DMTr)-protected nucleoside phosphoramidites serve as the starting materials.[2] During the coupling reaction the DMTr protecting group ensures that only one phosphoramidite reacts with the growing oligonucleotide chain. Before coupling of the next phosphoramidite, the acid-labile DMTr group is removed by treatment with a solution of an organic acid (e.g., dichloroacetic acid, trichloroacetic acid) in an organic solvent (e.g., toluene, dichloromethane)[3] followed by an acetonitrile wash. (Scheme 1) This reaction is a crucial step impacting both yield and quality of the final product.[4] Incomplete DMTr removal leads to formation of process-related impurities, which are shorter than the parent compound and are difficult to remove during purification. On the other hand, extended acid exposure leads to reduced yields and increased levels of failure sequences caused by depurination of purine nucleotides and subsequent chain cleavage during base deprotection with aqueous ammonium hydroxide.[5] In promoting the development of new synthetic oligonucleotides from the initial small laboratory scale to scales suitable for clinical trials to commercial-scale manufacturing there is a need to develop robust technologies that allow reproducible preparation of high-quality oligonucleotides at high yield.

On small-scale oligonucleotide synthesis (<100 μmol) silica-based controlled-pore glass (CPG) support in sparged reactor vessels is widely used. With increasing scale (production scale is approaching 1 mol) the use of packed-bed reactors (resembling chromatography columns), using tightly packed polystyrene-based support combined with flow-through mode of reagent and solvent delivery, allows for a more economical manufacture of oligonucleotide products. Resistance in the solvent delivery lines, bed height, and packing and swelling properties of the polymeric support in the reactor vessel (column design) lead to substantially higher backpressure compared to sparged reactors. To obtain good synthesis results it is crucial to maintain appropriate flow rates of reagent especially during DMTr removal and during the subsequent washing step. During synthesis of 20-mer

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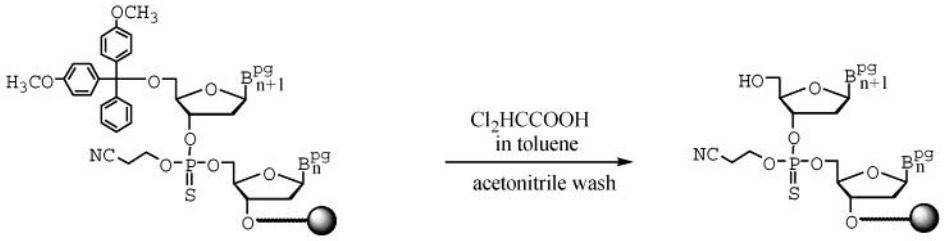
Scheme 1



pg = protecting group
B_n = nucleotide at position n
(counting from the 3' end)

phosphorothioate oligodeoxyribonucleotides, we consistently observe a significant increase in backpressure as the synthesis progresses. This may be explained by the increase in mass of the growing oligonucleotide and the concomitant decrease in void volume.[6] Switching from detritylation solution to wash solvent (acetonitrile) frequently causes brief pressure spikes that often exceed the maximum system pressure, resulting in shutdown of the automated synthesis. Furthermore, the high pressure used to create optimal flow rates may reduce the life of pumps and seals. The resistance of the synthesis column places a limitation on the flow rates employed or on the bed height (synthesis scale) that can be used with a particular system configuration. Therefore, we thought that further optimization of the flow rate/backpressure profile would increase flexibility and improve efficiency and robustness of automated solid-phase synthesis. The combination of swellable polystyrene/divinylbenzene-based support and inert, nonswellable filling materials was explored and compared to standard synthesis methods.[7] This contribution describes the application of glass microspheres and quartz sand as two examples of inert filling materials in packed-bed solid-phase synthesis columns as a means to reduce overall system pressure and pressure build-up during synthesis of oligonucleotides.[8]

ISIS 2503, a 20-mer phosphorothioate oligodeoxyribonucleotide (5′-PS-TCCGTCATCGCTCCTCAGGG)[9] was synthesized on an ACTA Oligo Pilot synthesizer (AmershamPharmaciaBiotech) on 1 mmol scale. Polystyrene/divinylbenzene-based Primer Support 200 dG was used as the solid support and as the source of the 3′-terminal nucleotide. Standard reagents [5′-*O*-DMTr and base-protected (benzoyl adenine, benzoyl cytosine, isobutyryl guanine) deoxyribonucleoside phosphoramidites (0.2 M in acetonitrile), 1*H*-tetrazole (0.45 M in acetonitrile) as activator, phenylacetyl disulfide (0.2 M in 3-picoline/acetonitrile)[10] as sulfur-transfer reagent, pyridine/*N*-methyl imidazole/acetic anhydride for capping] were used. For DMTr removal we used dichloroacetic acid in toluene (10%, v/v).[3b]

The flow rate for DMTr removal was 92 mL/min, and the flow rate for the subsequent acetonitrile wash was 120 mL/min. In the control synthesis (Syn 1) we packed a suspension of 23 mL (dry volume) of Primer Support 200 dG in acetonitrile in the Fineline 35 reactor column. In an effort to reduce backpressure (Syn 2) we packed a slurry of Primer Support 200 dG (23 mL, dry volume) and glass microspheres (23 mL, 10−95 μm, Duke Scientific) as inert filler material. Both solids were transferred into the synthesis column, anhydrous acetonitrile (200 mL) was added, and the mixture was vigorously shaken for about a minute to obtain a slurry with an even distribution of both types of particles. Then, the top piston was quickly compressed to pack the solid support in the synthesis column. Non-uniform packing of the solid-phase material may lead to preferential flow (channeling) resulting in inferior synthesis results. Syn 1 and Syn 2 were performed using identical synthesizer settings (reagent and solvent volumes, reagent equivalents, flow rates). In Syn 2 the column volume setting was the same as in Syn 1, and no adjustment was made for the amount of glass microspheres that was added to the solid support. ISIS 2503 was synthesized, and the pressure for detritylation and subsequent rinse was recorded after each coupling step.

In Figure 1, the pressure of Syn 1 (continuous line) during detritylation is represented by the crosses, and the corresponding acetonitrile wash, by triangles. The pressure during detritylation increased only slightly (ca. 1 bar) over the course of the synthesis from 11 to about 12 bar. However, the pressure during acetonitrile wash following detritylation increased more dramatically from 13 to about 19 bar. Manual intervention at wash steps after couplings 13 and 14 was required to restart the synthesis program as the maximum system pressure (20 bar) was temporarily exceeded during the wash (data points for couplings 13 and 14 are not included in graph), and the synthesis was paused automatically. Pausing the synthesis during the detritylation/acetonitrile wash step increases the time of the acid exposure of the oligonucleotide product, resulting in reduced yield and quality of product. In addition, manual intervention was necessary to continue the synthesis.

(6) On 1 mmol scale, the initial weight of support packed in the column is 5 g (0.2 mmol/g nucleoside loading). After 19 synthesis cycles, the weight of the support typically increases by ca. 8 g.
(7) In LC columns the backpressure is inversely proportional to the square of the particle diameter.
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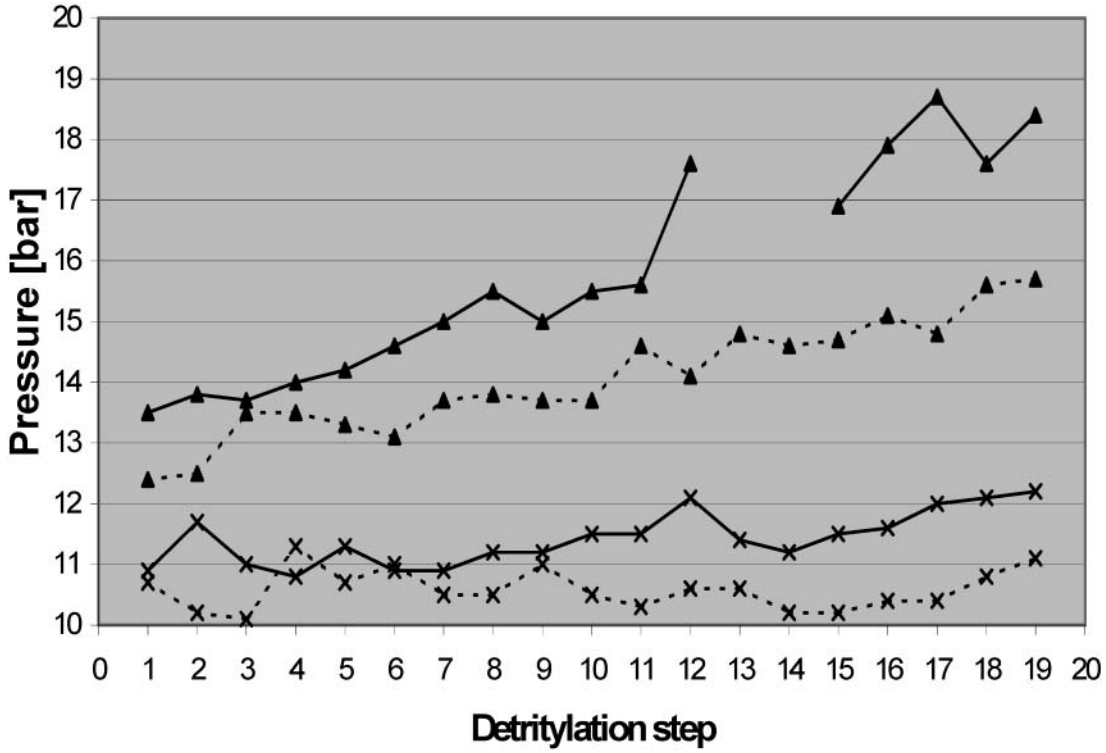

Figure 1. **Comparison of system pressure during detritylation (crosses) and acetonitrile wash (triangles) in the presence (Syn 2, dashed line) and absence (Syn 1, continuous line) of glass microspheres as inert filling material. The flow rates were 92 mL/min during detritylation and 120 mL/min during acetonitrile wash. In the absence of inert filling material the maximum pressure (20 bar) was exceeded during wash cycles 13 and 14.**

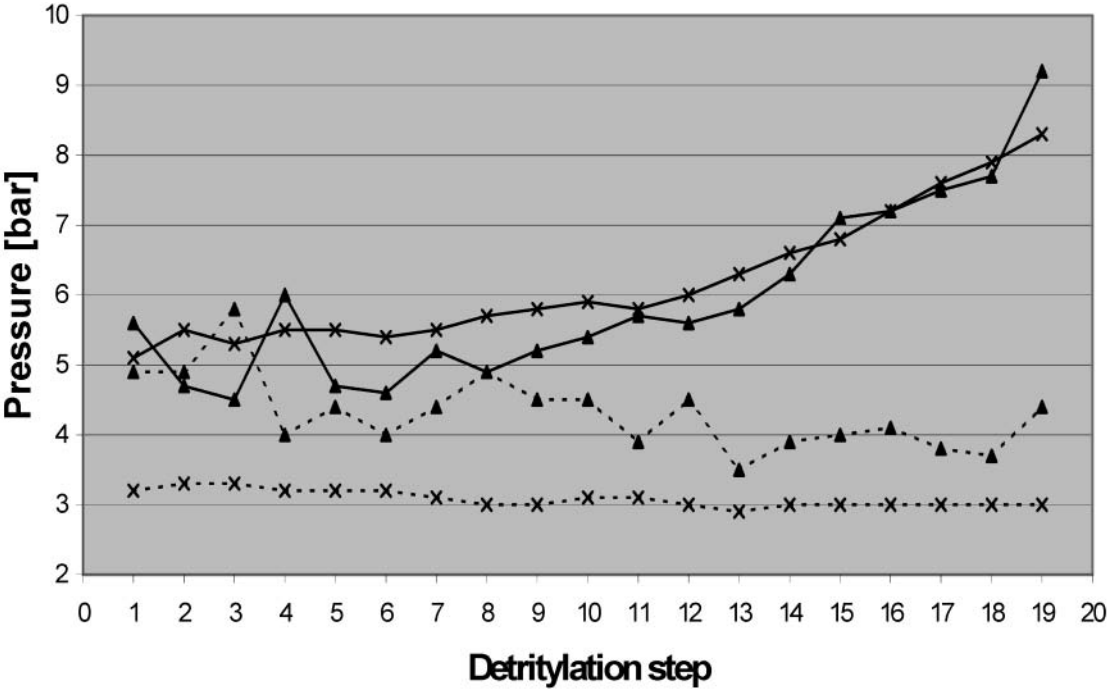

Figure 2. **Comparison of system pressure during detritylation (crosses) and acetonitrile wash (triangles) in the presence (Syn 4, dashed line) and absence (Syn 3, continuous line) of quartz sand as inert filling material. The flow rate during detritylation was 52 mL/min, during the acetonitrile wash, 60 mL/min.**

In contrast, the pressure during detritylation of Syn 2 (with an inert filler, dashed line) remained constant throughout the synthesis at ca. 11 bar. More importantly, the pressure during the wash cycle only increased from 13 to ca. 15 bar, without triggering overpressure shutdown of the synthesis. Most importantly, both yield and quality of the oligonucleotide product synthesized by both methods were comparable with the apparent advantage of Syn 2 that manual intervention was not necessary.

In a second set of experiments we intended to lower the backpressure even further, and we also wanted to test the hypothesis that using larger particles as filling materials

would allow the synthesis to proceed without any significant pressure increase.[7] White quartz sand (20 mL, −50 to +70 mesh, 210−300 μm, Aldrich) which is cheap and readily available, was packed together with Primer Support 200 dG (5 g, 1 mmol) in the Fineline 35 column. The pressure profile of this packing was compared to the pressure profile of a control synthesis (Syn 3) without inert filling material. (Figure 2). The flow rates were lowered (compared to the first set of experiments using glass microspheres) to 52 mL/min during detritylation and to 60 mL/min during acetonitrile wash to avoid manual intervention due to pressure spikes. As shown in Figure 2, the backpressure for detritylation and wash almost doubles over the course of the synthesis without the inert filling material (from ca. 4−5 to ca. 8−9 bar). In sharp contrast, the backpressure stays at a constant and significant lower level (3−5 bar) throughout the synthesis in which quartz sand was used as inert filling material (Syn 4). Syn 3 and Syn 4 were performed using identical synthesizer settings (reagent and solvent volumes, reagent equivalents, flow rates). In Syn 4 the column volume setting was the same as in Syn 3, and no adjustment was made for the amount of quartz sand that was added to the solid support. Yields and impurity profile of the oligonucleotide products from both syntheses were identical. This example further illustrates how optimizing backpressure-controlling factors can dramatically reduce column pressure without sacrificing performance.

In conclusion, we have shown that adding inert filling materials, e.g., glass microspheres or quartz sand, to the solid-support packing during oligonucleotide synthesis reduces system backpressure and backpressure buildup during the course of the synthesis. This technique enables oligonucleotide synthesis at larger scales (higher bed height) and at higher flow rates necessary for preparation of high-quality products. Furthermore, flow rate adjustments and manual interventions are avoided. Despite the increase in column volume by adding inert filling materials, there is no need for using increased amounts of reagents and solvents. The addition of inert filling materials to the standard solid support offers an inexpensive way to increase lab productivity.

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